Neurovascular Factors in Wound Healing in the Foot Skin of Type 2 Diabetic Subjects

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OBJECTIVE — Delayed wound healing in diabetic patients without large-vessel disease has been attributed to microvascular dysfunction, neuropathy, and abnormal cellular and inflammatory responses. The role of these abnormalities has mainly been examined in animal models. Few studies have been undertaken in diabetic patients, and those that have are limited due to analysis in wounds from chronic ulcers. In this study, we quantified the rate of wound healing in relation to skin neurovascular function and structure following a dorsal foot skin biopsy in type 2 diabetes.

RESEARCH DESIGN AND METHODS — Twelve healthy control subjects and 12 type 2 diabetic subjects with neuropathy but without macrovascular disease were studied. We quantified rate of wound healing and related it to skin microvascular function (laser Doppler imager $[LDI]_{max}$), blood vessel density, small nerve fiber function (LDI_{flare}) and nerve fiber density, vascular endothelial growth factor (VEGF) and its receptor (FLK1), and hypoxia-inducible factor (HIF)-1α expression.

RESULTS — The rate of wound closure was identical between control subjects and diabetic patients despite a significant reduction in maximum hyperemia (LDI_{max}), epidermal and dermal VEGF-A, and epidermal and dermal blood vessel VEGFR-2 expression as well as the neurogenic flare response (LDI_{flare}) and dermal nerve fiber density. There was no significant difference in HIF-1α and dermal blood vessel density between control subjects and diabetic patients.

CONCLUSIONS — In conclusion, the results of this study suggest that wound closure in subjects with type 2 diabetes is not delayed despite significant alterations in neurovascular function and structure.

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Wound healing is impaired in diabetic patients and has been attributed to both macro- and microvascular disease leading to tissue hypoxia, peripheral neuropathy, and abnormal cellular and inflammatory pathways predisposing to infection in foot ulcers (1–4). The molecular basis for these abnormalities has been examined mainly in animal models, which have a limited translational capacity.

The loss of protective sensation due to neuropathy and diminished trophic effect by neuropeptide deficiency have been proposed to lead to trauma and increased pressure on the foot skin and a diminished hyperemic response to tissue injury, respectively (5). Furthermore, these alterations may lead acute wounds to advance to chronic wounds with impaired healing (6). More recently, small fiber dysfunction has been shown to be an early feature in patients with type 2 diabetes and has also been implicated in delayed wound healing (7,8). Moreover, several microvascular abnormalities, including a reduced response to tissue injury causing underperfusion, the development of dependent edema due to a defective venoarteriolar reflex, and increased permeability of capillaries, have also been proposed to delay wound healing (9,10). Most human studies have shown no reduction in skin capillary density, suggesting that microvascular function may be sufficiently abnormal to reduce tissue blood flow without an actual reduction in overall vascular density in those with diabetes (11,12).

The molecular basis for these alterations has not been studied in detail in patients with diabetes. Few studies on wound healing have been undertaken in diabetic patients, and those to date have been limited to chronic ulcers. In diabetic animals, a reduction in IGF-I, IGF-II, keratinocyte growth factor, and platelet-derived growth factor (13) occurs, and application of these growth factors normalizes wound healing (14). Matrix metalloprotienases are increased in chronic ulcers in diabetic patients and in animal models of diabetes (15). Recently, the expression of vascular endothelial growth factor (VEGF), which promotes angiogenesis, has been shown to be reduced in the skin wounds of diabetic animals, and topical VEGF improved wound healing (16,17). Diabetic wounds in animal models also show abnormal angiogenesis and a reduction in the expression of nerve growth factor and its receptors. Nerve growth factor, in addition to its neurotrophic properties, has been shown to be proangiogenic, and nerve growth factor supplementation improves vascular regeneration via VEGF-A to accelerate wound healing (18,19).

In this study, we quantified the rate of wound healing in acute ulcers following a punch skin biopsy from the dorsum of the foot in diabetic patients and control subjects. This was examined in relation to skin microvascular function (laser Doppler imager $[LDI]_{max}$), blood vessel density, and expression of VEGF and its receptor (VEGFR)-2, and hypoxia-inducible factor (HIF)-1α. C-fiber function (LDI_{flare}) and dermal nerve fiber density were also quantified.

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Abbreviations: HIF, hypoxia-inducible factor; LDI, laser Doppler imager; VEGF, vascular endothelial growth factor; VEGFR, receptor of VEGF.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

RESEARCH DESIGN AND METHODS

— Twelve healthy control subjects (C group) and 12 subjects with type 2 diabetes and neuropathy (D group) were studied. Subjects were recruited on a consecutive basis from the diabetes outpatient clinics of the Ipswich Diabetes Centre. All subjects with diabetes selected for this study had peripheral neuropathy, as impaired wound healing is typically associated with this complication. Subjects with clinical features of peripheral vascular disease (ankle brachial pressure index <0.8) were excluded. The study was approved by the local ethical committee, and all subjects gave informed consent to take part in the study.

Assessment of neuropathy

Neuropathy was assessed by measurement of the vibration perception threshold, using the ascending method of limits. A mean of three values was taken for analysis. The results were expressed in volts. A vibration perception threshold of ≥15 V (i.e., >95th percentile) for this age-group was considered abnormal (20).

In addition, sensation was assessed using the Neuropen (Owen Mumford, Oxford U.K.), which contains a 10-g monofilament to assess pressure perception and a Neurotip (Owen Mumford) for pinprick sensation. Ten-gram monofilaments were applied for 2 s on the plantar aspect of the first, third, and fifth metatarsal heads, and Neurotip was applied at the epinychium of the first toe (i.e., a total of four sites were tested, three for the 10-g monofilament and one for Neurotip). At sites where sensation was not felt, the test was repeated three times to confirm the abnormality. Subjects were assigned to have impaired sensation if they could not feel a stimulus on more than one of the tested sites. All diabetic subjects recruited had absent ankle reflexes, impaired sensation using the Neuropen, and impaired vibration perception threshold.

Assessment of LDI_{flare}

Subjects were allowed to acclimatize for 30 min in a temperature-controlled room, where the temperature was maintained at 25 ± 1°C. The foot temperature was measured proximal to the first and second metatarsal heads using an infrared thermometer (Linear Laboratories, Fremont, CA). Room temperature and relative humidity were monitored throughout. The axon-reflex–mediated LDI_{flare} was examined using an LDI (Moor Instruments, Devon, U.K.) (8). This uses a stable helium neon gas laser ($\lambda = 632.8$ nm) beam that is deflected by a moving mirror to create a raster pattern across the surface of the skin. The Doppler shifted light from moving blood, and nonshifted light from static tissue is directed back via the same mirror into two detectors. Fluctuations in the wavelength are processed to calculate the flux that is proportional to tissue blood flow. The data were recorded to a computer using MoorLDI (version 3.11) software, and a flux image was produced using a palette of 16 equally spaced colors in which dark blue represented lowest perfusion and red the highest perfusion.

The skin proximal to the first and second metatarsal heads on the dorsum of the foot was heated with a circular skin heater (diameter −0.9 cm) (Moor Instruments) to 44°C for 20 min. An area of 3.5 × 3.5 cm surrounding the heated skin was scanned with the LDI aligned to be perpendicular to the dorsum of the foot at a fixed distance of 30 cm, immediately after removing the heater probe. The scan images were stored in a computer and processed offline. On the flux image, the region of interest demarcated by the edge of the flare was drawn, and the area of the LDI_{flare} was calculated using MoorLDI (version 3.11) software. The results were expressed in centimeters squared.

Assessment of maximum hyperemia (LDI_{max})

The same flux image described above was also used to calculate the maximum hyperemia. A region corresponding exactly to the size of heater probe was defined, and the mean flux within that region was calculated using MoorLDI (version 3.11) software. This is the maximum hyperemic response that we have termed LDI_{max}. The results are expressed in arbitrary perfusion units.

Skin biopsy

Skin biopsies were performed using a sterile 3-mm biopsy punch (Stiefel Laboratories, Bucks, U.K.) in the same area in which the LDI_{flare} had been assessed on a separate day. No local anesthetic was applied and all subjects tolerated the biopsy. There was no infection or other adverse event.

Assessment of wound closure

Wound closure was assessed by digital microscopy at magnification ×50 immediately after biopsy, day 3, and day 10. Digital photographs were stored in the computer, and the wound area was analyzed offline using Mouseyes software. The computer monitor was calibrated and the region of interest drawn along the circumference of the wound to enable calculation of wound area expressed in millimeters squared.

Immunohistochemistry

The skin biopsy specimen was immediately fixed in 4% paraformaldehyde for 18–24 h, routinely processed (Citadel 2000 Processor; ThermoElectron, Waltham, MA), and embedded in paraffin wax. Serial 5-μm tissue sections were cut from each block (Microtome Leitz Wetzlas 1512) and mounted onto positively charged slides (Fisher Scientific, Loughborough, U.K.). Sections were dewaxed in xylene and gradually rehydrated through decreasing ethanol dilutions. Epidermal melanin was bleached with 0.25% $KMnO_4$ followed by 5% oxalic acid. Series selected for blood vessel density assessment by CD31/vWF immunolocalization underwent trypsinization. For VEGF-A and VEGF-R2, sections were microwaved to disclose the antigen, and, for HIF-1α, optimal visualization was obtained using a tyramide amplification reagent (CSA I; Dako). Sections were incubated overnight at 5°C with mouse monoclonal antibodies to CD31 and vWF (diluted 1:100 and mixed) (both from Dako) and to VEGFR-2 (1:50; Santa Cruz Biotechnology) and with rabbit polyclonal antibodies to VEGF-A (1:300; Santa Cruz Biotechnology) and to HIF-1α (1:300; Abcam). For nerve fiber density, sections were incubated overnight with 1:1,200 biogenesis polyclonal rabbit anti-human antibody (Serotec, Oxford, U.K.). Biotinylated swine anti-rabbit secondary antibody (1:300, 1 h) was then applied; sections were quenched with 1% H_2O_2 in 30% MeOH-PBS (30 min) before incubation for 1 h with 1:500 horseradish peroxidase streptavidin (Vector Laboratories, Peterborough, England). The reactions were demonstrated using the following, listed sequentially: biotinylated secondary antibodies, streptavidin horseradish peroxidase, and the chromogenic substrate 3′-3′diaminobenzidine (DAB; Sigma-Aldrich, Dorset, U.K.).

Analysis of staining

Patterns of immunostaining were examined by light microscopy. To quantify the amount of VEGF-A, VEGF-R2, and HIF-1α staining, microphotographs were taken using a Nikon digital camera mounted on a Leitz DM RB microscope.

Table 1—*Subject characteristics*

	C group	D group
n	12	12
Age (years)	50.2 (56.0–62.2)	54.0 (55.0–61.5)*
Duration (years)	—	10.0 (5.8–14.8)
BMI (kg/m^2)	25.40 (22.9–27.4)	32.3 (30.6–34.8)†
A1C (%)	—	8.8 (8.4–9.1)
Ankle brachial pressure index	1.1 (1.0–1.2)	1.2 (1.0–1.3)*
Vibration perception threshold (V)	7.0 (4.3–8.0)	40.7 (23.7–51.0)‡
10-g monofilament/pressure perception	Normal	Abnormal

Data are median (interquartile range). *No significant difference between the groups; †$P = 0.01$; ‡$P < 0.0001$.

Percentages of stained area were quantified separately in the epidermis and in the upper dermis with the computer program Leica QWin Standard, version 2.4 (Leica Microsystem Imaging, Cambridge, U.K.), set to detect color intensities within a fixed, constant range. Blood vessel and nerve fiber cross-sections in the papillary dermis were counted manually and their density expressed as number per millimeter squared.

Statistical analysis

Descriptive statistics (median and interquartile range) were used to describe subject characteristics. Mann-Whitney *U* test was used to determine the differences between the groups. Mean ± SD for each variable is described, and a *P* value of <0.05 was considered significant. SPSS (version 11.0) software package was used for statistical analysis.

RESULTS

Clinical characteristics of diabetic and control subjects are shown in Table 1. All subjects were Caucasian, and there was no significant difference in age between groups C and D.

Wound closure

Wound closure (Fig. 1) determined by area change (mean ± SD) did not differ between diabetic patients (in mm^2: day 0, 6.17 ± 0.5; day 3, 4.63 ± 0.4; and day 10, 2.93 ± 0.5) and control subjects (day 0, 6.28 ± 0.3; day 3, 4.89 ± 0.8; and day 10, 3.01 ± 0.7). There were no complications, and all wounds were fully reepithelialized by day 10.

Neurovascular function/structure

LDI_{max} expressed as perfusion units (PU) was significantly reduced in the diabetic group (C: 577.4 ± 125.3 vs. D: 310.33 ± 97.3; $P < 0.0001$), whereas dermal blood vessel density (per mm^2) did not differ between control subjects (116.5 ± 21.0 mm^2) and diabetic patients (116.8 ± 27.8 mm^2) ($P = 0.96$). LDI_{flare} was significantly reduced in diabetic patients compared with control subjects (in cm^2: C: 5.2 ± 1.8 vs. D: 1.8 ± 0.7; $P < 0.0001$) as was dermal nerve fiber density (per mm^2: C: 456.3 ± 160.1 vs. D: 216.0 ± 144.0; $P = 0.001$). LDI_{flare} was significantly associated with nerve fiber density ($r = 0.6$; $P < 0.0001$).

Vascular factors

The expression of HIF-1α in epidermal vessels (C: 6.42 ± 6.32 vs. D: 8.68 ± 11.74; $P = 0.63$) and dermal vessels (C: 16.99 ± 15.98 vs. D: 10.22 ± 12.55; $P = 0.14$) did not differ significantly between control subjects and diabetic patients (Table 2). However, there was a significant difference in the expression of epidermal VEGF-A (C: 0.36 ± 0.30 vs. D: 0.16 ± 0.18; $P = 0.03$) and dermal VEGF-A (C: 0.04 ± 0.07 vs. D: 0.01 ± 0.004; $P = 0.04$). Also, epidermal blood vessel VEGFR-2 (C: 21.58 ± 25.99 vs. D: 9.66 ± 12.91; $P = 0.05$) and dermal blood vessel VEGFR-2 (C: 7.94 ± 6.88 vs. D: 3.45 ± 3.14; $P = 0.04$) expression were significantly reduced in diabetic patients compared with control subjects (Table 2, Fig. 2).

CONCLUSIONS

The pathophysiological mechanisms contributing to delayed wound healing in diabetes are complex and may be mediated by vascular, neuronal, cellular, and immune factors. Our study is unique, as we have quantified the wound-healing response and related it to neurovascular integrity and the expression of vascular factors central to the wound-healing response.

Against expectation and in contrast to findings in animal models and the observation of poor healing in diabetic patients with foot ulceration, the rate of wound closure was identical in diabetic and control subjects. It is important to note that we studied the healing response of an acute wound on the dorsum of the foot in an area that is not exposed to continued high pressure that occurs in chronic diabetic plantar foot ulcers. Whether acute wounds on the plantar

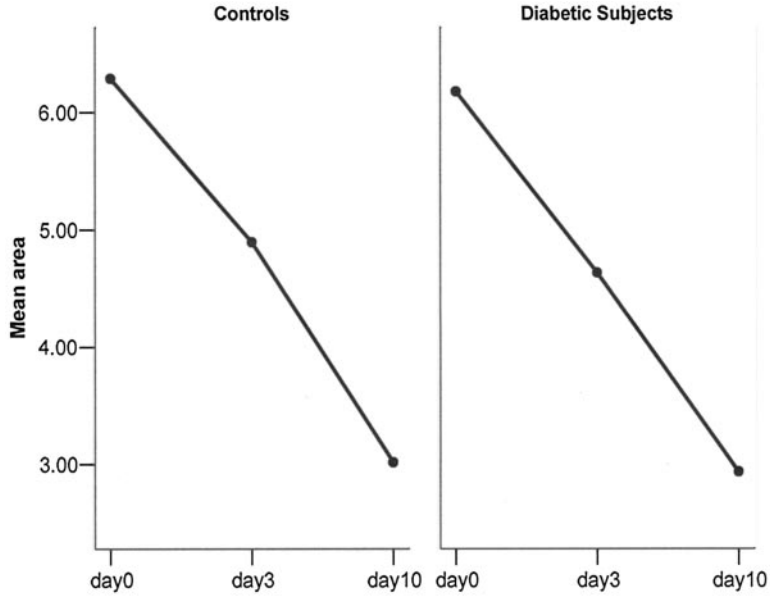

Figure 1—*Wound area from biopsy to day 10 to assess rate of closure expressed as means ± SD in mm^2. No significant difference between the control and diabetic groups (day 0:* P = *0.78; day 3:* P = *0.56; day 10:* P = *0.95).*

Table 2—*Neurovascular factors in wound closure*

	C group	D group	*P* value
LDI_{max} (PU)	577.4 ± 125.3	310.33 ± 97.3	<0.0001
LDI_{flare} (cm^2)	5.2 ± 1.8	1.8 ± 0.7	<0.0001
Nerve fiber density (per mm^2)	456.3 ± 160.1	216.0 ± 144.0	0.001
Blood vessel density (per mm^2)	116.5 ± 21.0	116.8 ± 27.8	0.96
VEGF (epidermal)	0.36 ± 0.30	0.16 ± 0.18	0.03
VEGF (dermal)	0.04 ± 0.07	0.01 ± 0.004	0.04
VEGFR-2 (epidermal)	21.58 ± 25.99	9.66 ± 12.91	0.05
VEGFR-2 (blood vessel)	7.94 ± 6.88	3.45 ± 3.14	0.04
HIF-1α (epidermal)	6.42 ± 6.32	8.68 ± 11.74	0.63
HIF-1α (blood vessel)	16.99 ± 15.98	10.22 ± 12.55	0.14

Data are means ± SD. Maximum hyperemia: LDI_{max} (PU), C-fiber function: LDI_{flare} (cm^2), and dermal nerve fiber density: nerve fiber density (per mm^2) was significantly reduced in diabetic patients compared with control subjects. Epidermal and dermal VEGF-A and epidermal and dermal blood vessel VEGFR-2 expression were significantly reduced in diabetic patients. HIF-1α and dermal blood vessel density did not differ significantly between the diabetic patients and control subjects.

surface behave differently or trigger factors such as infection, which could turn such wounds into chronic ulcers, remains to be determined. None of the wounds in the present study became infected, and great care was taken to ensure that the wounds were well protected.

Impaired hyperemic response to tissue injury and iontophoresis of acetylcholine in the presence of normal vascular density has led previous investigators to implicate functional microvascular defects in delayed wound healing in diabetic patients (21–23). However, this mechanism has only been inferred and never previously directly assessed.

While Veves et al. (24) previously demonstrated a reduction in endothelial nitric oxide synthase expression, few studies have explored in detail other molecular alterations that may be relevant to the wound-healing response following injury in diabetic patients. We believe such studies are essential if we are to gain an understanding of any perturbation in the wound-healing response following injury and development of an ulcer. It is known that VEGF expression is normally increased during the granulation phase of wound healing, and this response is diminished in diabetic mice (16). Furthermore, topical application of VEGF or overexpression of VEGF by an adenoviral vector markedly accelerates wound healing in diabetic animals (17,25). While adenovirus-mediated gene transfer of a soluble form of VEGFR-2 (Flk-1) reduces angiogenesis, it does not delay wound closure in *db/db* mice (26). Although tissue hypoxia, a typical feature of healing wounds, is thought to increase the expression of VEGF through HIF-1α (27), the role of HIF-1α in diabetic wounds has not been explored in experimental studies and in particular in diabetic patients. In the present study, we demonstrate no difference in HIF-1α expression per se between diabetic patients and control subjects.

Thus, despite an impaired maximal hyperemic response, wound healing was normal in our diabetic patients. Blood vessel density was similar in the control and diabetic groups, consistent with our previous findings in those with type 1 (28) and type 2 (24) diabetes. The normal vascular density may well have maintained skin oxygenation, as evidenced by comparable HIF-1α expression in both groups. Despite lower expression of VEGF and VEGFR-2 in diabetic skin, wound closure did not differ between diabetic patients and control subjects. This suggests that VEGF may play a limited role in acute wound healing in diabetic patients.

With regard to neuropathy, it may contribute to the development of foot ulceration via a loss of protective sensation and reduced axon reflex–mediated vasodilatation. Impaired expression and regulation of nerve growth factor and reduction in skin nerve density have been speculated to delay healing (29). We demonstrate a marked reduction in both dermal nerve fiber density and the axon reflex as assessed by LDI_{flare}. However, despite significant abnormalities in both parameters there was no impact on wound healing.

One of the perceived limitations of this study is that of studying an acute

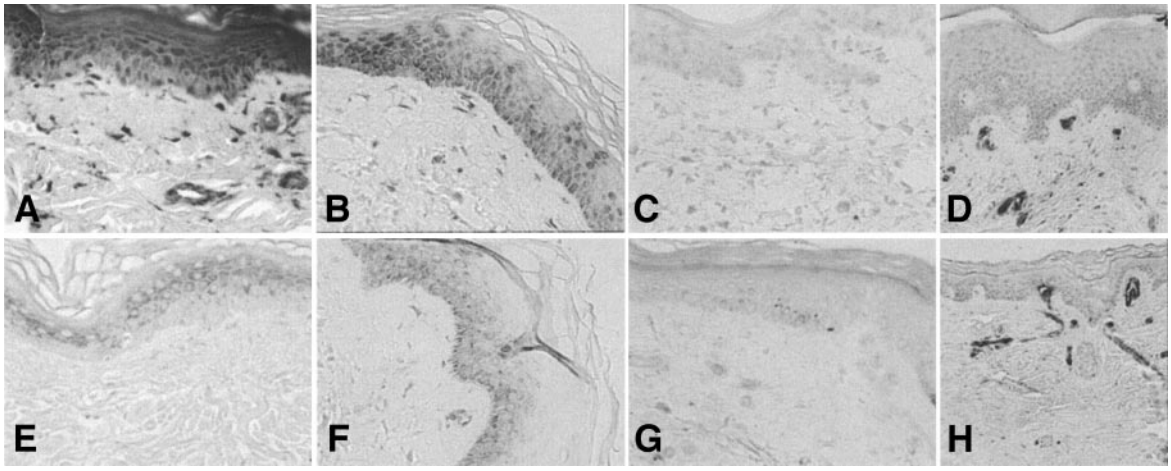

Figure 2—*The picture shows immunostaining for VEGF-A* (A *and* E)*, VEGFR-2* (B *and* F)*, HIF-1α* (C *and* G)*, and blood vessels* (D *and* H)*. The first row* (A–D) *contains normal case subjects. The second row* (E–H) *contains diabetic case subjects. Note less pronounced epidermal staining for VEGF-A and VEGFR-2 in the diabetic case compared with the corresponding control subject.*

wound and expression of neuronal and vascular integrity at baseline, with healing by secondary intention compared with chronic wounds in a typical diabetic foot ulcer. However, this is no different than all experimental studies where wounds are also acute and yet the wound healing response is delayed. Thus, we believe our study has provided important translational insights and questioned established concepts of wound healing mainly derived from studies in experimental animals. This study also establishes the safety of distal skin biopsies in the assessment of diabetic neuropathy. Due to the relatively small number of study subjects, further larger studies may be needed to confirm the findings.

In conclusion, this study suggests that wound closure in subjects with type 2 diabetes is not delayed despite significant alterations in neurovascular function and structure. This reiterates the importance of pressure relief in those with neuropathic ulcers, restoration of adequate blood flow in those with ischemic ulceration, and aggressive treatment of wound infection as the principal strategies to successfully heal diabetic wounds.

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